



The protective effect of polyphyllin I on myocardial ischemia/reperfusion injury in rats

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Background: Myocardial ischemia/reperfusion (I/R) injury has become a global public health concern. An increasing amount of evidence has shown that polyphyllin I (PPI) has anti-apoptotic and antioxidant functions. This study was performed to evaluate the cardioprotective effects of PPI in a rat model of myocardial I/R injury and the underlying mechanism.

Methods: We exposed induced a rat model of I/R injury by exposing rat hearts to left anterior descending coronary artery ligation for 30 min, followed by 24 h of reperfusion. Cardiac function was analyzed by echocardiography and HE staining. Myocardial apoptosis, inflammation, and oxidative stress were detected to analyze the PPI's role in I/R injury.

Results: The results showed that pretreatment with PPI improved impaired histological morphology, as shown by histopathological examination. Echocardiography analysis showed that PPI increased the levels of HR, left ventricular ejection fraction (LVEF), and left ventricular wall thickness (LVWT), accompanied by decreased left ventricular end-systolic volume (LVESV). Also, PPI decreased the expression of CK-MB, Mb, cTnI, and LDH. Specifically, PPI also changed the expression of apoptotic makers (Caspase-3, Bax, and Bcl-2), inflammatory cytokines (TNF- α , IL-6, iNOS, and IL-10) and oxidative stress markers (SOD, GSH, ROS, and MDA). Notably, western blot (WB) showed that PPI treatment inhibited the phosphorylation activity of NF- κ B p65.

Conclusions: The findings showed that PPI exerted a favorable protective effect on I/R injury by inhibiting the inflammatory response and oxidative stress. It offered new drug candidates for the treatment of myocardial I/R injury.

Keywords: Polyphyllin I (PPI); oxidant; apoptosis; inflammation; myocardial ischemia-reperfusion

Submitted Mar 26, 2020. Accepted for publication May 12, 2020.

doi: 10.21037/atm-20-3371

View this article at: <http://dx.doi.org/10.21037/atm-20-3371>

Introduction

Currently, acute myocardial infarction (AMI) is a common cardiovascular disease, which leads to high morbidity and mortality around the world. For patients with AMI, the most prompt and effective treatment is to improve

myocardial ischemic and restrict the size of myocardial infarction. As far as we know, myocardial reperfusion is so far the principal or only strategy for the treatment of AMI, which is clinically achieved by angioplasty or thrombolytic therapy, thereby promptly restoring blood

supply (1). However, this sudden reperfusion should result in secondary cascade damages, called ischemia/reperfusion (I/R) injury, which paradoxically deteriorated ischemic damages and further swelled infarct size (2). Also, myocardial I/R injury could trigger a large variety of pathological changes, including local acute inflammatory reactions, metabolic disorders, and cell apoptosis or necrosis, which ultimately led to cardiac dysfunction (3). Simultaneously, the pathogenesis of myocardial I/R injury is involved. It involves multiple molecular processes, such as reactive oxygen species (ROS) accumulation (4), calcium ion overload (5), inflammatory cytokines influx (6), and mitochondrial permeability transition pore (mPTP) openings (7), among many others. Although there are many necessary research reports on myocardial I/R injury, the clinical effects are not satisfactory. We still need further research to find more effective clinical treatments.

Chinese herbal medicine has a history of thousands of years in the treatment of multiple diseases. Polyphyllin I (PPI), also called Chong Lou saponin I, a steroidal saponin, is a bioactive substance separated from the rhizome of *Paris polyphylla*. Previous reports revealed that PPI possessed an anti-cancer effect in a variety of cancers through inhibiting the proliferation and growth of tumor cells (8-12), thereby being used as a possible anti-cancer drug candidate. Besides, Shi *et al.* found that PPI could promote the occurrence of protective autophagy in HCC cells (13). Yang *et al.* reported that PPI inhibited cell survival capability and eased apoptosis in gefitinib-resistant NSCLC cells and xenograft models (14).

Furthermore, PPI could permeabilize the membrane of human red blood cells (RBC), resulting in increased Ca^{2+} concentration (15), and PPI also directly triggered mitochondrial swelling (16), thus causing cell apoptosis. Previously, PPI was reported to down-regulate the constitutive phosphorylation of NF- κ B p65 protein and its downstream target genes expression in hepatocellular carcinoma (17). Similarly, recent studies revealed that PPI also protects against myocardial I/R injury, remarkably inhibiting the secretion of inflammatory cytokines and suppressing the translocation of p65 (18,19). At present, many drugs have been demonstrated to be effective against reducing myocardial I/R injury in animal models but not involved in clinical practice (1,20,21), and the role of PPI in myocardial I/R injury is still ambiguous.

In this study, we established a model of myocardial I/R in rats, the aim was to investigate the protective effect of PPI

on myocardial injury, and also explore the role of PPI on I/R-induced myocardial inflammation and oxidative stress, enriching its pharmacological actions. We present the following article in accordance with the ARRIVE reporting checklist (available at <http://dx.doi.org/10.21037/atm-20-3371>).

Methods

Primary materials

PPI powder (molecular formula: $C_{44}H_{70}O_{16}$, molecular weight: 855.017, density: 1.4 ± 0.1 g/cm³, purity $\geq 98\%$) was obtained from Sichuan Weikeqi Biological Technology Co., Ltd. (Chengdu, China). Male Sprague-Dawley (SD) rats (≥ 6 weeks old, 251–300 g) were bought from Zhejiang Chinese medical university Laboratory Animal Research Center (Zhejiang, China).

Primary assay and methods

Animals administration

All male SD rats were placed in the same environment (23 ± 2 °C, relative humidity $55\% \pm 5\%$, and a 12 h light/dark cycle), kept food and water-free. The rats were randomly grouped as follows (n=9): sham group, I/R group, I/R + PPI (50 mg/kg) group, and I/R + PPI (100 mg/kg) group, I/R + PPI (150 mg/kg) group. The sham operation group did not have I/R but was given normal perfusion at the same time. Rats in the sham group and I/R group were given 0.9% physiological saline, yet in I/R + PPI treatment groups were given different doses of PPI (22) by intraperitoneal administration (once a day for 14 days), followed by I/R surgery.

Animal model of myocardial I/R

Besides, all the rats were fasting for 12 h before the operation, then they were anesthetized with 10% chloral hydrate and fastened on the operating table, yet the I/R procedures were achieved referring to previous descriptions (23). Briefly, open the left chest to reveal the heart, peel the pericardium to find the coronary artery, and ligate the left anterior descending coronary (LAD). LAD ligation induced myocardial ischemia for 30 minutes and reperfusion for 120 minutes—and the same surgery for sham operation without LAD ligation. Twenty-four hours after I/R surgery, cardiac tissues, or blood samples were collected and stored

(-80 °C) for further analysis.

All animal experiment steps were conducted following the National Institutes of Health (NIH) guide for the care and use of laboratory animals [8th edition, National Academies Press (US), 2011] and approved by Chengdu University of Traditional Chinese Medicine.

Echocardiography

SD rats were narcotized by intraperitoneal injection of 2% isoflurane and placed in a flat position. The echocardiography measurement of left ventricular function was done by digital ultrasound equipment Vevo 2100 (VisualSonics, Ontario, Canada) and 18 MHz transducers after cardiac perfusion. Then, M-mode recordings of the left ventricular ejection fraction (LVEF), left ventricular wall thickness (LVWT), and left ventricular end-systolic volume (LVESV) were calculated. All the results were averaged from at least three consecutive cardiac cycles measuring from the M-mode images and were analyzed by a blinded researcher.

Histopathological analysis

Rat heart tissues were excised and fixed in 10% formalin buffer. After paraffin embedding, the tissues sections were chipped to 4 µm thickness and then stained with hematoxylin-eosin (HE) liquid for the histopathological examination.

Immunohistochemistry (IHC) assay

The rat heart tissues were fixed in 10% buffered formalin, embedded in paraffin, and sectioned at a thickness of 4 µm according to standard procedures. Next, deparaffinized sections were then hydrated. Furthermore, endogenous peroxidase activity was blocked by Hydrogen Peroxide Block for 15 min. After antigen retrieval in 10 mM heated citrate buffer for 10 min, then the sections were incubated with primary antibody: anti-anti-Caspase-3 antibody (ab4051, 1:500, Abcam, UK) overnight at 4 °C. Subsequently, the corresponding secondary antibody was incubated for 1 h at room temperature. Finally, the specimens were stained with DAB kit (Boster Biological Technology Co., Ltd., Wuhan, China). Then, the specimens were washed with PBS, and images were captured under confocal microscopy (Leica, Wetzlar, Germany).

TUNEL staining

Rats were euthanized, and paraffin sections were prepared as above method. TUNEL assay was conducted using a

TUNEL kit (R&D Systems, Minnesota, USA), according to the manufacturer's protocols. Five fields of vision were randomly selected under an optical microscope (Leica, Wetzlar, Germany), positive brown cells and total cells were counted, and the apoptosis rate was calculated.

Determination of inflammatory cytokines

Blood samples were centrifuged for 10 min at 2,000 G, and supernatants were collected, the serum levels of TNF- α , IL-6, iNOS, and IL-10 were detected using ELISA kits, according to the manufacturer's protocols (Thermo Fisher, Waltham, MA, USA). The results at 450 nm was recorded and the sample concentration calculate was calculated with the best fitting curve for data analysis.

Oxidative stress detection

After I/R, blood samples were collected from the left common carotid artery and centrifuged for 10 min at 2,000 G, the levels of SOD, ROS, MDA, and GSH in the serum were measured with professional test kits from Nanjing Jiancheng Bioengineering Institute (Nanjing, China), and all operating procedures were performed according to the manufacturer's instructions.

Determination of cardiac injure biomarkers

The myocardial damage was evaluated by measuring the level of myocardial-specific markers, including AST, CK-MB, cTnT and LDH. myocardial perfusion injury is accompanied by changes in the content of some biomarkers. Therefore, changes in serum levels of biomarkers can reflect tissue damage. according to the manufacturer's instructions, we tested the expression of cTnI, CK-MB, Mb and LDH according to the instructions provided in the purchased kits from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

Western blot (WB) analysis

The total protein samples were extracted with RIPA lysis buffer (Beyotime Institute of Biotechnology, Haimen, China). Protein lysates were quantified using a Pierce™ BCA protein assay kit (23227, Thermo Fisher, Waltham, MA, USA) according to the manufacturer's protocols. About 50 µg of proteins loaded onto 12% SDS PAGE gels, All membranes were blocked using 5% nonfat dry milk in Tris-buffered saline (TBS) at 4 °C for 30 min, and were incubated with the following primary antibodies: anti-Ki67 antibody (ab16667, 1:500, Abcam, UK), anti-Surviving antibody (ab134170, 1:500, Abcam, UK), anti-

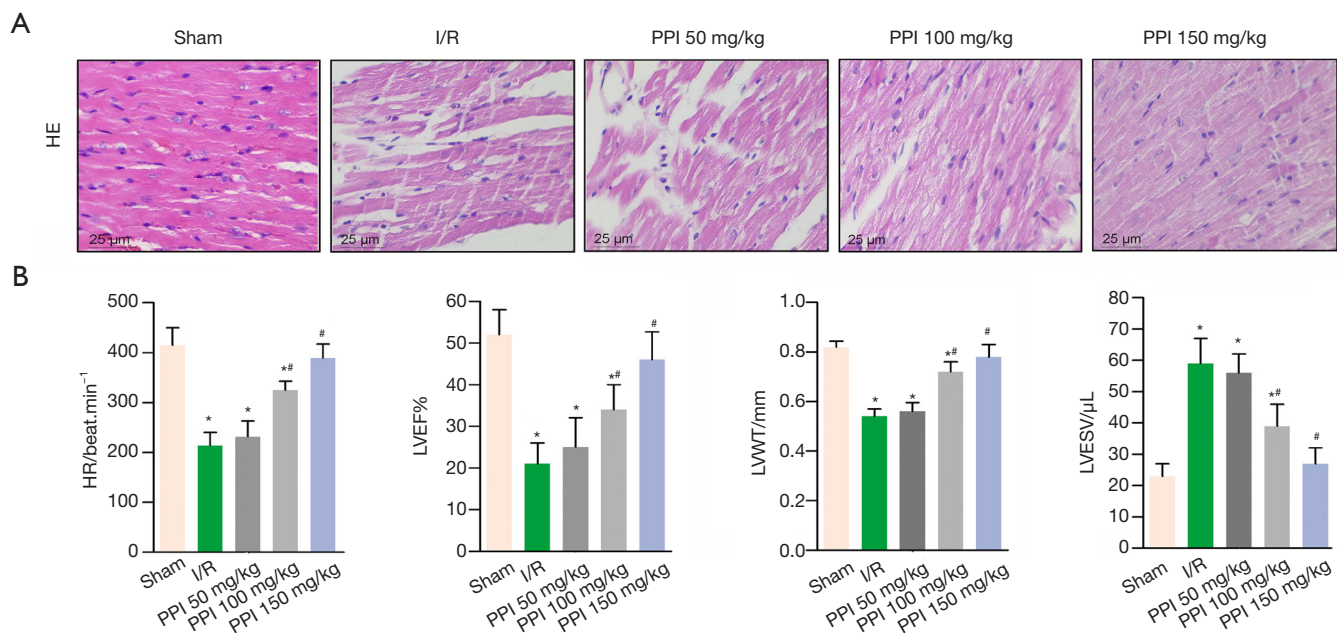


Figure 1 PPI prevented cardiac dysfunction. (A) After 24 h of reperfusion, myocardial histology was detected by HE staining; representative images were taken under 200 \times magnification. (B) Left ventricular ejection fraction (LVEF), left ventricular wall thickness (LVWT) and left ventricular end-systolic volume (LVESV) were assessed by echocardiography. Data are presented as the mean \pm SD (n=10). *, P<0.05 vs. control group, #, P<0.05 vs. I/R group. All independent experiments were repeated at least three times. PPI, polyphyllin I; I/R, ischemia/reperfusion.

Bax antibody (ab32503, 1:500, Abcam, UK), and anti-Bcl-2 antibody (ab196495, 1:500, Abcam, UK), anti-NF- κ Bp65 antibody (ab16502, 1:500, Abcam, UK) and anti-NF- κ Bp65 (phosphor S536) antibody (ab86299, 1:500, Abcam, UK). Next, the membranes were incubated with a horseradish peroxidase-conjugated secondary antibody, respectively. Finally, the bands were exposed to ECL reagent (Thermo Fisher, Waltham, MA, USA). β -actin was used as the loading control.

Statistical analysis

All the experiments were conducted at least three times, and the data were expressed as mean \pm SD. Statistical analysis was conducted using IBM SPSS Statistics 25.0. (IBM, Armonk, USA). Student's *t*-test was used to evaluate the difference between the two groups, and one-way ANOVA was used to analyze the significance of the difference among different treatment groups. P<0.05 showed statistical significance.

Results

PPI alleviated pathological injury of cardiac tissue and prevented the cardiac function

The myocardial sections were stained via HE staining to assess the myocardial injury. As showed in *Figure 1A*, the sham group displayed no apparent difference in the morphological structures, whereas the I/R group showed obvious myocardial fiber fracture, cellular edema, necrosis, and inflammatory infiltration. Interestingly, different doses of PPI (50, 100, 150 mg/kg) significantly attenuated myocardial tissue injury in the myocardium compared with I/R group. Following, we used echocardiography analysis to investigate cardiac function during acute myocardial I/R injury. As showed in *Figure 1B*, I/R treatment significantly decreased HR, LVEF, and LVWT, accompanied by increased LVESV, respectively. However, PPI remarkably improved the cardiac function compared with the I/R group.

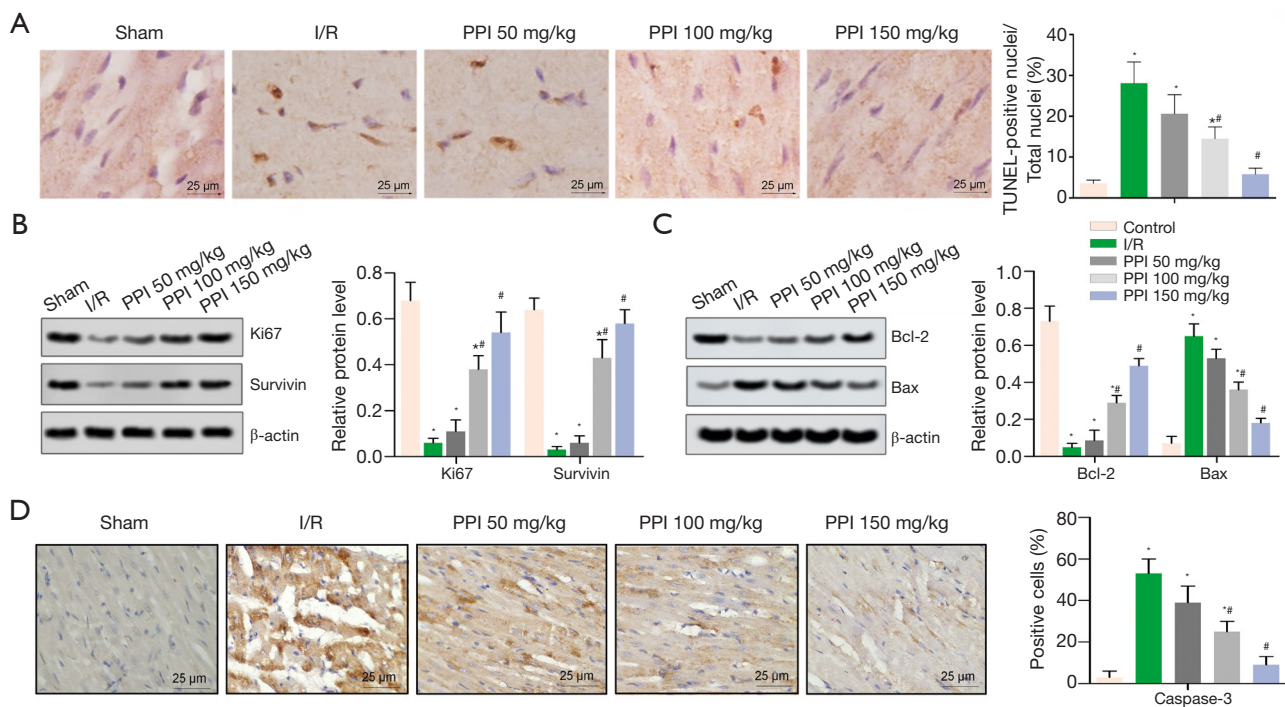


Figure 2 PPI attenuated myocardial injury after I/R. (A) TUNEL staining for myocardial tissues from different groups, representative images were taken under 200 \times magnification. (B,C) The protein levels of Ki67, Surviving, Bax, and Bcl-2 were measured by western blot assay. (D) Positive cells of Caspase-3 representative images that were taken under 200 \times magnification. Data are presented as the mean \pm SD (n=10). *, P<0.05 vs. control group, #, P<0.05 vs. I/R group. All independent experiments were repeated at least three times. PPI, polyphyllin I; I/R, ischemia/reperfusion.

PPI attenuated apoptosis after I/R

TUNEL staining demonstrated the anti-apoptotic effect of PPI (50, 100, 150 mg/kg) in myocardial I/R rats (Figure 2A). Parallely, the protein levels of Ki67 and Surviving were down-regulated in the I/R group (Figure 2B). As expected, PPI reversed the change, compared with I/R group. Furthermore, we evaluated the expression of Bcl-2, Bax, and caspase-3 proteins by WB and IHC assay, respectively. According to our results, the levels of caspase-3 and Bax were up-regulated, the level of Bcl-2 was down-regulated by I/R. Contrarily, PPI significantly down-regulated the levels of caspase-3 and Bax, while up-regulated the level of Bcl-2, compared with I/R group (Figure 2C,D).

PPI reduced myocardial injury and oxidative stress in rats

To further evaluate IR-induced myocardial injury, we measured the release of several biomarkers (cTnI, CK-MB, Mb, and LDH) in serum. Figure 3A showed the expression

of cTnI, CK-MB, Mb, and LDH were increased in I/R group, compared with sham group. On the contrary, PPI (50, 100, 150 mg/kg) reversed the results of I/R processing. Next, we investigated the serum levels of oxidative stress makers. Figure 3B showed the expression of SOD and GSH were markedly increased, and the expression of ROS and MDA were decreased after I/R. PPI significantly increased antioxidant levels of SOD and GSH and decreased oxidant levels of ROS and MDA in rats with myocardial I/R injury, compared with I/R group.

PPI inhibited I/R-induced inflammatory response and impaired the activity of NF- κ B p65

To determine the role of PPI in inflammation, we evaluated the serum levels of TNF- α , IL-6, iNOS, and IL-10 via ELISA assay. As showed in Figure 4, I/R remarkably up-regulated the levels of TNF- α , IL-6, iNOS while down-regulated IL-10. On the contrary, the protein levels of TNF- α , IL-6, and iNOS were effectively up-regulation,

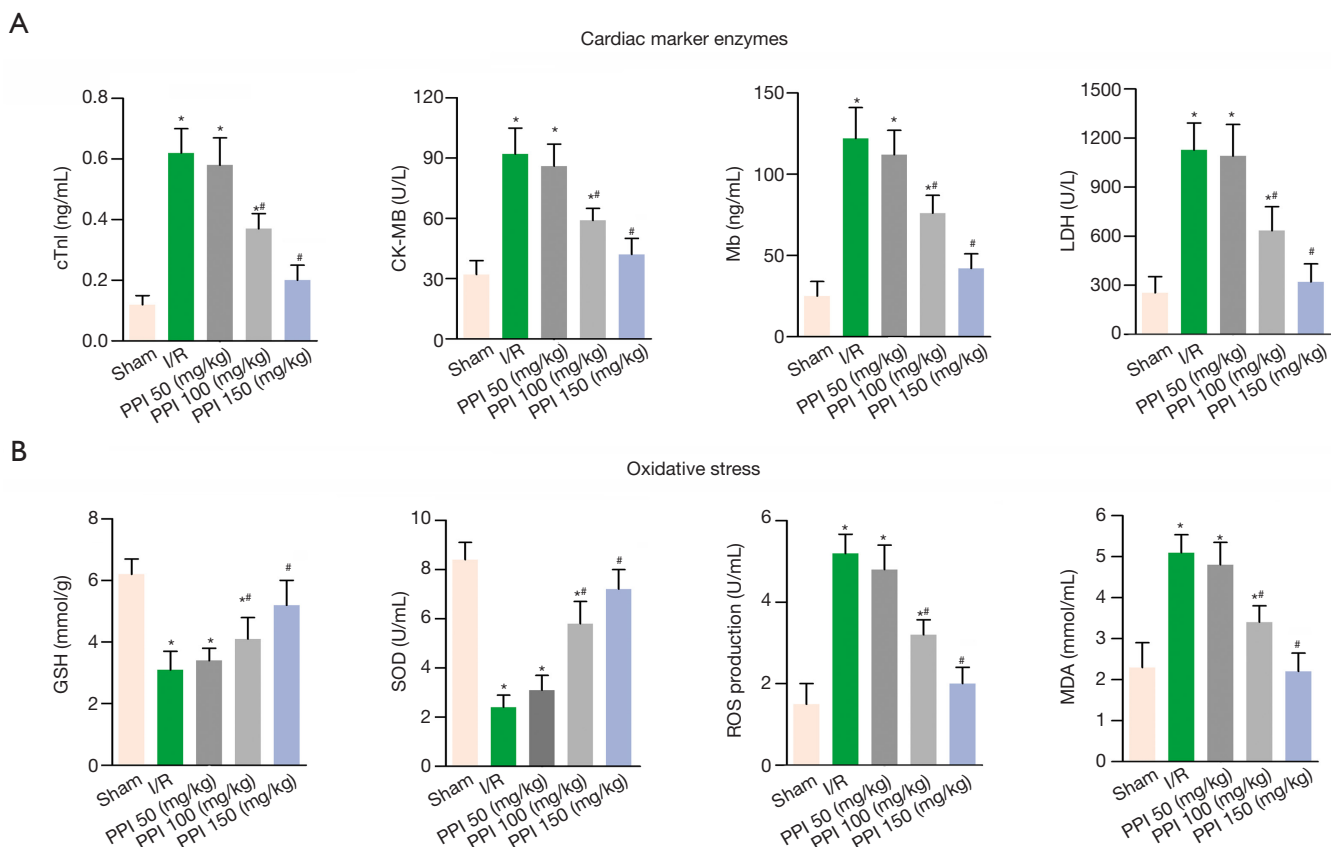


Figure 3 PPI reduced oxidative stress in rats. (A) Effects of PPI treatment on cTnI, CK-MB, and Mb, and LDH levels of serum, respectively. (B) Effects of PPI treatment on GSH, SOD, ROS, and MDA levels of serum, respectively. Data are presented as the mean \pm SD (n=10). *, P<0.05 vs. control group, #, P<0.05 vs. I/R group. All independent experiments were repeated at least three times. PPI, polyphyllin I; I/R, ischemia/reperfusion.

and IL-10 was down-regulation in the PPI groups (50, 100, 150 mg/kg), compared with I/R group (Figure 4A,B,C,D). Therefore, these data showed that PPI could effectively attenuate I/R-induced inflammation. Moreover, WB analysis showed that p-p65/p65 dramatically lifted in the IR group, and treatment with PPI inverted the ratio of p-P65/P65 (Figure 4E,F).

Discussion

Studies show that I/R injury induces free radical formation and inflammation within hours, leading to excessive production of oxidants and inflammatory mediators (24). Oxidative stress and inflammation are vital processes in the pathogenesis of myocardial I/R injury (25). In the present study, PPI significantly increased the levels of SOD and GSH (antioxidant makers) and decreased levels of ROS,

and MDA (oxidant makers). Besides, myocardial I/R causes cytokines such as TNF- α , IL-6, and IL-1 β . In this, as an important cytokine in inflammation, IL-6 plays an important role in I/R induced injury, which is a downstream target of IL-1 β . TNF- α secreted by macrophages promotes inflammatory cascade by increasing the release of other proinflammatory cytokines and influencing the recruitment of neutrophils (26). Basing on these findings, we found that PPI significantly decreased the expression of TNF- α , IL-6, and iNOS, increased IL-10 expression. These results, together, were consistent with the pathological evaluation of myocardial tissues.

Ischemia triggers apoptosis of cardiomyocytes and then expands and partially contributes to cardiac death through reperfusion (27). Song *et al.* reported that I/R injury markedly induced activation of 12/15-LOX and resulted in cardiomyocyte apoptosis, increased caspase-3 activity,

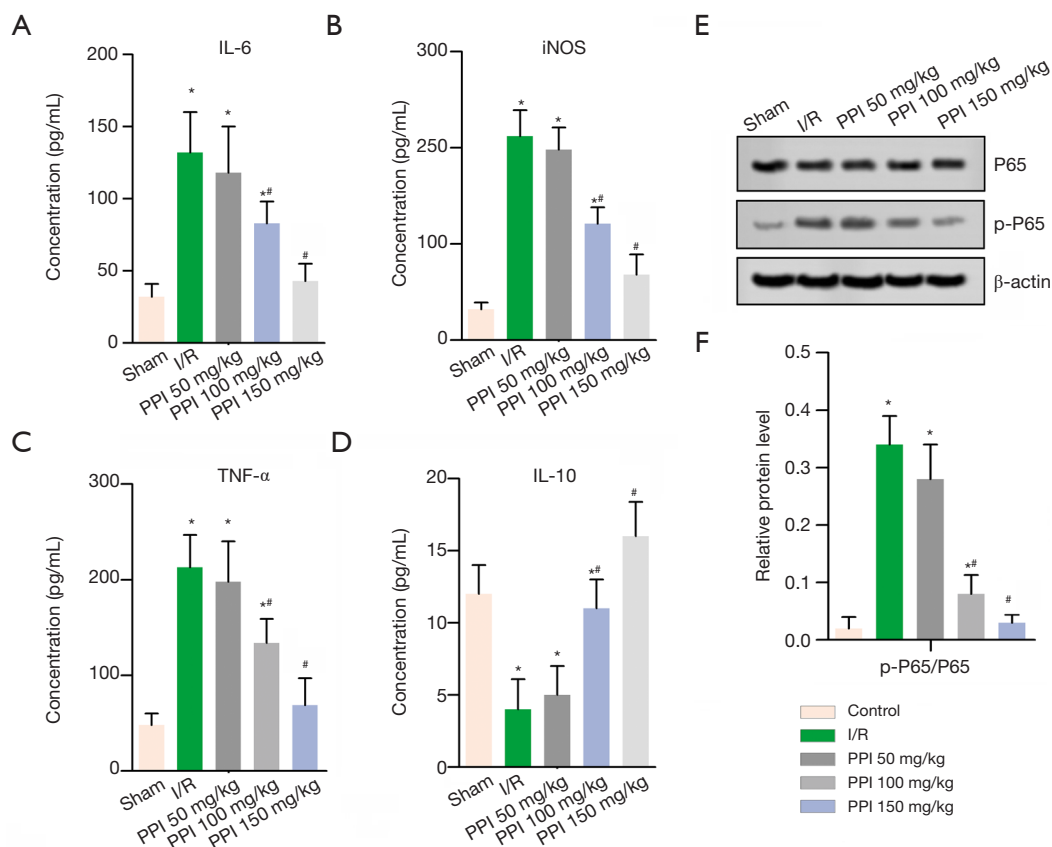


Figure 4 PPI inhibited I/R-induced inflammatory response. The serum levels of IL-6 (A), iNOS (B), TNF- α (C), and IL-10 (D) were measured by ELISA assay. (E,F) The protein levels of p65 and p-p65 were measured by western blot assay. Data are presented as the mean \pm SD (n=10). *, P<0.05 vs. control group, #, P<0.05 vs. I/R group. All independent experiments were repeated at least three times. PPI, polyphyllin I; I/R, ischemia/reperfusion.

and Bax/Bcl-2 ratio (28). Especially, cleaved caspase-3 was a crucial triggering factor (29), which activated various downstream proteases, such as cysteine protease, inhibiting cell proliferation, and inducing apoptosis. In our study, TUNEL staining and the detection of Caspase-3, which were the apoptosis markers, revealing the number of apoptotic cells were decreased and the expression of caspase-3 was down-regulated by PPI treatment. Besides, the expression of Bcl-2 was decreased and Bax was increased. Interestingly, we also found PPI decreased the levels of injury bio-markers (cTnI, CK-MB, Mb, and LDH) in serum, and up-regulated Ki67 and Surviving protein levels, suggesting PPI attenuated myocardial injury via mediating myocardial apoptosis in rats.

NF- κ B pathways take part in many physiological functions, including hypertension, heart failure, and myocardial hypertrophy. NF- κ B, as a redox-sensitive

transcription factor, is usually present in the cytoplasm as a heterodimer. The prototypical signals, such as LPS, IFN- γ , IL-1, and TNF- α , could induce phosphorylation of I- κ B protein by upstream kinases (IKK), which resulted in the ubiquitination and degradation of I- κ B, thereby releasing the NF- κ B and further activating NF- κ B (19). Many studies showed that NF- κ B is activated after myocardial I/R injury (20,30). Therefore, inactivation of the NF- κ B pathway in cardiomyocytes has cardioprotective effects against I/R-induced myocardial oxidative stress injury, cell death, and other injuries (31). Our study showed PPI inhibited the activity of NF- κ B p65 (22). Ulteriorly, active NF- κ B then translocates into the nucleus and activates downstream target genes, which experience ROS-mediated activation, stimulating the transcription of several protein mediators, for example, proinflammatory cytokines and pro-fibrotic response that activate several cell death pathways (32).

Our results showed that PPI protects myocardial injury mediated NF- κ B pathway by inhibiting the inflammatory response and oxidative stress contradicting these reports. Nevertheless, this is still to be investigated in the future.

Conclusively, this study demonstrated PPI alleviated myocardial I/R injury, inhibited myocardial apoptosis, and weakened the inflammation response and oxidative stress following I/R. Also, PPI mediated the activity of NF- κ B p65. Hence, the findings suggested PPI existed a protective role of myocardial I/R injury in rats, and it supplied a new vision for I/R treatment.

Acknowledgments

Funding: None.

Footnote

Reporting Checklist: The authors have completed the ARRIVE reporting checklist. Available at <http://dx.doi.org/10.21037/atm-20-3371>

Data Sharing Statement: Available at <http://dx.doi.org/10.21037/atm-20-3371>

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <http://dx.doi.org/10.21037/atm-20-3371>). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The Ethics Committee of Chengdu University of Traditional Chinese Medicine approved all animal experiments.

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Cite this article as: Huang R, Shu J, Dai X, Liu Y, Yu F, Shi G. The protective effect of polyphyllin I on myocardial ischemia/reperfusion injury in rats. *Ann Transl Med* 2020;8(10):644. doi: 10.21037/atm-20-3371